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The Isolation of a Suppressible Nonsense Mutant in Mammalian Cells

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Summary

An HGPRT$^-$ cell line derived from mouse L cells has been shown to have the following properties: it is CRM$^+$; the defective HGPRT molecules are altered in the carboxyterminal peptide; the mutant cells regain HGPRT activity when ochre-suppressor tRNA is microinjected into them, but not when amber-suppressor or wild-type tRNAs are injected. We conclude from these properties that this mutant cell line contains an ochre nonsense mutation (UAA) in the structural gene for HGPRT.

Introduction

The isolation and characterization of nonsense mutants and nonsense suppressors in mammalian cells should provide a valuable new tool for genetic analysis of mammalian cells and their viruses. For example, the classification of a mutation as suppressible by a nonsense suppressor provides a strong criterion that the mutation is in a structural gene. This inference can be made because of the very nature of nonsense mutants and their suppressors. A nonsense mutation generates an in-phase, polypeptide-chain-termination codon (UAA, UAG or UGA) in the interior portion of a structural gene. As a consequence of this mutation, an aminoterminal polypeptide fragment, rather than the completed polypeptide chain, is synthesized (Sarabhai et al., 1964). In bacteria and yeast, suppressors of nonsense mutations arise from mutations in tRNA genes which permit the mutant tRNA to translate a termination codon as an amino acid codon (Capecchi and Gussin, 1965; Engelhardt et al., 1965; Goodman et al., 1968; Capecchi, Hughes and Wahl, 1975; Gesteland et al., 1976). Thus in the presence of a suppressor tRNA, the completed polypeptide product of a nonsense mutant can be synthesized. If the amino acid inserted at the site of the mutation does not markedly alter the protein structure, then an active gene product may be restored.

Nonsense mutants are clearly conditional lethal mutations, since the physiological effect of the nonsense mutation can be studied in the presence and absence of the suppressor. It is of practical importance that this should be a very stringent conditional lethal system in mammalian cells. The expectation of stringency is based upon the following considerations: most NH_2 terminal polypeptide fragments should not exhibit biological activity; such fragments are probably rapidly degraded in mammalian cells (Capecchi et al., 1974); and cell-free protein synthesis experiments indicate that the level of "read-through" of a nonsense mutation in the nonpermissive system is less in mammalian cell-free extracts than in the comparable bacterial extracts (Capecchi et al., 1975).

The question of stringency of this potential conditional-lethal system is an important one, since application of the other common conditional-lethal system (temperature-sensitive mutations) to mammalian cell studies has met with some technical difficulty. The versatility of temperature-sensitive mutants has been limited because mammalian cells cannot be grown over as wide a temperature range as bacteria or yeast. As a result of this narrow range, many temperature-sensitive mutants isolated in mammalian cells and their viruses are "leaky"—that is, they exhibit measurable activity at the nonpermissive temperature.

A further important property of the nonsense/nonsense-suppressor system is that the affected mutant gene product can often be identified, since one can usually distinguish between the mutant polypeptide fragment and the suppressed complete polypeptide chain.

The strategy which we adopted to search for nonsense mutants and their suppressors in mammalian cells was to start by isolating a large number of mouse L cell lines deficient for the nonessential enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT; E.C. 2.4.2.8). This enzyme catalyzes the conversion of the purines hypoxanthine and guanine to their respective nucleotides IMP and GMP. It was selected as the target enzyme for these studies for a number of reasons. The enzyme can be readily assayed in cell-free extracts and in intact cells. Selective methods for isolating cell lines having lost or regained enzymatic activity had been developed (Szybalski, Szybalska and Ragni, 1962; Littlefield, 1963). Purine analogues, such as 8-azaguanine and 6-thioguanine, kill cells containing HGPRT and select for resistant cell lines which have reduced HGPRT activity. Such mutants remain viable because purine nucleotides can be synthesized by de novo pathways in the absence of HGPRT. Revertants, which have regained HGPRT activity, can be selected by blocking de novo purine biosynthesis with methotrexate and simultaneously supplying hypoxanthine. Such treatment renders the cells dependent upon HGPRT for synthesis of purine nucleotides from hypoxanthine.

Because it seemed probable that most nonsense mutants would exhibit very little enzymatic activity, the purine analogue concentration (8-azaguanine

plus 6-thioguanine) use for isolating the HGPRT$^-$ cell lines was chosen so that all the surviving clones contained <0.1% of the parental HGPRT activity.

These mutant cell lines were then divided into two classes: those which had lost enzymatic activity but still retained protein which cross-reacted with antisera prepared against purified HGPRT (CRM$^+$), and those which had lost both enzymatic activity and immunological cross-reactivity (CRM$^-$). The cross-reacting material from the CRM$^+$ mutants could be analyzed by standard methods of protein chemistry to determine whether the CRM exhibited smaller subunit molecular weights relative to the parental HGPRT molecules. If certain CRMs appeared to be fragments, one could further ask whether the alteration occurred at the carboxyterminal end of the polypeptide chain as predicted for nonsense mutants. The CRM$^-$ cell lines, which were expected to contain most of the HGPRT$^-$ nonsense mutants, were more difficult to analyze. Two separate approaches were used. First, the CRM$^-$ cell lines were tested for sensitivity to phenotypic suppression by microinjection of suppressor tRNA (to be described). Second, assuming that the collection of CRM$^-$ mutants contained suppressible nonsense mutants, revertants from each of the CRM$^-$ mutant cell lines were isolated and tested in vitro for suppressor tRNA activity.

Results

Properties of the HGPRT$^-$ Cell Lines

The HGPRT$^-$ mutants used in this study were selected, after mutagenesis with nitrosoguanidine, for resistance to the purine analogues 8-azaguanine and 6-thioguanine (Sharp, Capecchi and Capecchi, 1973). As previously mentioned, each of these cell lines contains <0.1% of the HGPRT activity present in the parental mouse L cells. A specific antibody directed against highly purified mouse-liver HGPRT was used for detect CRM in these HGPRT$^-$ clones. Two methods for detecting CRM activity were used: a standard precipitation inhibition assay (Suskind, 1957) and a radioimmune precipitation assay (Wahl, Hughes and Capecchi, 1975). The latter assay proved to be much more sensitive for detection of altered HGPRT molecules. By these methods, 40% of the HGPRT$^-$ cell lines were shown to contain CRM. Since the amount of CRM varies among cell lines, the distinction of CRM$^+$ from CRM$^-$ lines must be an operational one, based on some arbitrarily chosen limit of detectable CRM. We designated a cell line as CRM$^+$ if it contained >1% of the amount of CRM present in the parental cell line in an equivalent assay. Examination of the physical properties of CRM from different mutant cell lines indicated that they arose from mutations at many different loci within the HGPRT structural gene (Wahl et al., 1975). The vast majority of these independently isolated CRMs did, however, have subunit molecular weights indistinguishable from wild-type HGPRT. In retrospect, this is not a very surprising result. We examined the rates of degradation of several missense mutants of HGPRT and found that they are selectively degraded 20–100 fold faster than the wild-type protein (Capecchi et al., 1974). These studies showed that even a small change in a protein, such as a missense mutation that leaves the protein still immunologically detectable, is sufficient to cause rapid selective degradation. Most nonsense mutants seem likely to be degraded even faster. Following this line of reasoning, the only nonsense mutants we might have expected to escape rapid degradation and be detected as CRM$^+$ were those resulting from the introduction of a nonsense codon very near the carboxyterminal end of the coding region. In fact, as shown in Figure 1, the HGPRT$^-$ CRM$^+$ mutant which we discuss in this manuscript is not resolved from wild-type HGPRT by electrophoresis in SDS-urea polyacrylamide gels. Nevertheless, we demonstrate that this mutant contains an altered carboxyterminal tryptic peptide, and that the chromatographic behavior and amino acid composition of this peptide are consistent with the fact that it is shorter than the corresponding wild-type peptide. For the experiment shown in Figure 1, mutant and wild-type cells were labeled with ^{35}S- and ^{3}H-methionine, respectively; extracts were prepared and immunoprecipitated with anti-HGPRT sera. The immune precipitates were then mixed and electrophoresed on SDS-urea polyacrylamide gels. The resolving power of this gel is approximately ±700 daltons (that is ± six amino acids).

Tryptic Peptide Analysis of Wild-Type and Mutant HGPRT

The ability to resolve differences between mutant and wild-type HGPRT is greatly enhanced by examination of their tryptic peptides. Furthermore, an analysis of the tryptic peptides should afford the opportunity to identify the carboxyterminal peptide. Since nonsense mutants generate NH_2 terminal polypeptide fragments, it is clearly important to look for changes in the carboxyterminal region of the protein.

The processing of the mutant and wild-type HGPRT for tryptic peptide analysis includes labeling the proteins in vivo with the desired radioactive amino acids, preparing extracts, immunoprecipitating the HGPRT molecules and purifying the immune precipitates by SDS-urea polyacrylamide gel

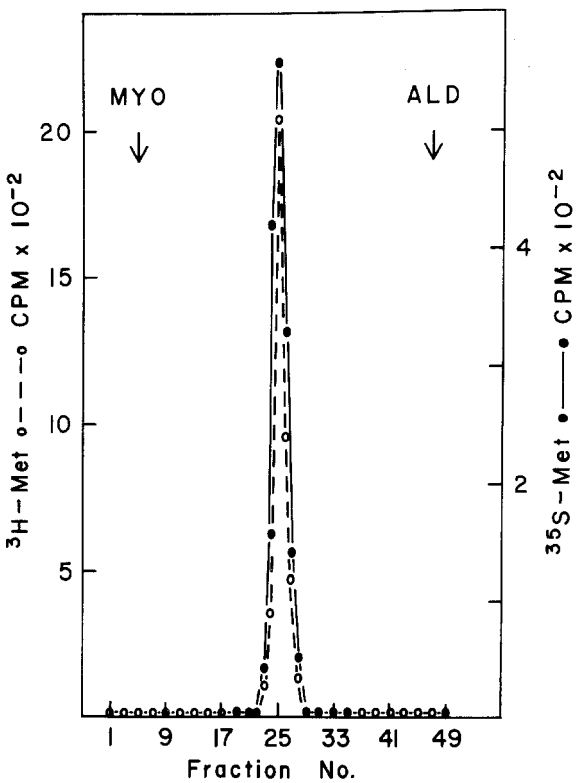

Figure 1. Analysis of HGPRT Isolated From L^+ and Mutant ($HGPRT^-$, CRM^+) Cell Extracts by Radioimmune Precipitation and SDS-Urea Polyacrylamide Gel Electrophoresis

The L^+ and mutant cell lines were labeled with ^{3}H- and ^{35}S-methionine, respectively; the cell extracts were prepared and treated with antiserum against HGPRT and antibody against rabbit IgG. The radioimmune precipitates were then mixed and subjected to electrophoresis in an SDS-urea polyacrylamide gel. The gel was internally standardized with fluoresceinated myoglobin (MYO) and aldolase (ALD).

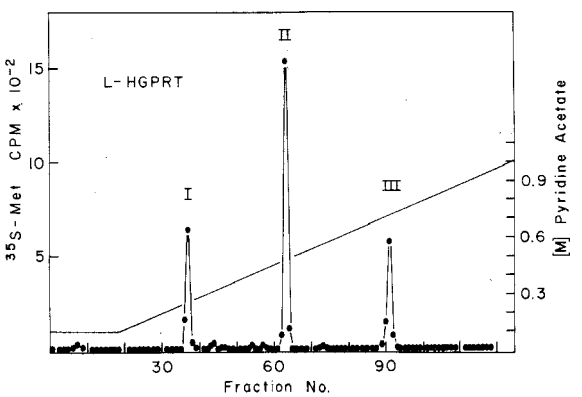

Figure 2. Analysis of the Methionine-Containing Tryptic Peptides of HGPRT by Cation-Exchange Chromatography

L^+ cells were labeled with ^{35}S-methionine. The labeled HGPRT was purified from the cell extract by immune precipitation and SDS-urea polyacrylamide gel electrophoresis. The HGPRT molecules eluted from the gel were digested with TPCK-treated trypsin, and the peptides were separated by chromatography in Bioradaminex 5 resin using a pyridine acetate gradient.

electrophoresis. The mutant and wild-type HGPRT are then eluted from their respective gels, mixed and trypsinized with enzyme that has been treated with the chymotrypsin inhibitor TPCK. The tryptic peptides are separated by cation-exchange chromatography using a procedure similar to that developed by Milman, Krauss and Olsen (1977) for analyzing the tryptic peptides of human HGPRT.

In Figure 2, we illustrate an experiment in which L^+ cells were labeled with ^{35}S-methionine. The profile of the methionine-containing tryptic peptides of HGPRT is relatively simple. Peptides I and III contain one methionyl residue, whereas peptide II appears to contain two. There is a fourth methionine-containing peptide (illustrated in Figures 5 and 6) which requires strong base to be eluted from the column. These results are consistent with the amino acid composition analysis of purified mouse-liver HGPRT, which indicated that the protein contains five methionyl residues per 27,000 daltons of protein (unpublished results).

In Figure 3, we compare the elution profile of the methionine-containing tryptic peptides of HGPRT isolated from the mutant, labeled with ^{3}H-methionine, and wild-type cells, labeled with ^{35}S-methionine. The mutant and wild-type HGPRT molecules were mixed prior to digestion with trypsin and then chromatographed on the cation-exchange column. Mutant peptide I elutes from the column before the corresponding wild-type peptide. We conclude from this that the mutant peptide is either shorter than, or more negatively charged than, the wild type peptide or both.

The tryptic peptide profile shown in Figure 4 is from an experiment similar to that shown in Figure 3, except that the radioactive labels were reversed. We observe again that the mutant peptide I elutes from the column ahead of the wild-type peptide.

If the mutant in question was a nonsense mutant, the prediction was that peptide I is the carboxyterminal tryptic peptide of HGPRT. That this was so is shown in Figures 5 and 6. Figure 5 shows that the methionine-containing peptides II and IV are lysyl tryptic peptides. Figure 6 shows that peptide III contains arginine. Peptide I, however, contains neither lysine nor arginine. Since trypsin specifically cleaves proteins *after* lysine and arginine, and peptide I contains neither, peptide I must be the carboxylterminal tryptic peptide of HGPRT.

We mentioned earlier that the mutant carboxyterminal peptide is either shorter than, or more negatively charged than, the corresponding wild-type peptide or both. We favor the interpretation that the mutant peptide is shorter for the following reasons. The pH of the column at the elution position of the altered peptide was sufficiently low that it could not

be more negatively charged by having acquired an acidic amino acid residue. Furthermore, it could not be more negatively charged by having lost a basic residue since the wild-type peptide did not contain lysine or arginine. Similarly, it could not be more negatively charged by having lost a histidine residue since neither the wild-type nor the mutant peptides contained histidine (see Figure 7). By elimination, therefore, we are left with the hypothesis that the mutant peptide is shorter than the wild-type one. Direct testing of this hypothesis will have to await sequence analysis.

These results are consistent with the hypothesis that this CRM$^+$ mutant is a nonsense mutant; however, a word of caution should be entered at this point. Showing that a mutant protein has a smaller subunit molecular weight than the wild-type protein and even showing that the alteration occurs at the carboxyterminal end of the protein is consistent with, but does not prove, that the mutation is a nonsense mutation. Alternative explanations include that the mutation is the result of a small internal deletion at the carboxyterminal end; that it is a frameshift mutation; or that it is an error in post-synthetic processing. Indeed, we believe that HGPRT is processed from a larger precursor. This is of interest since HGPRT does not appear to be a protein destined for export. Thus processing of mammalian proteins may be a much more general phenomenon than previously believed.

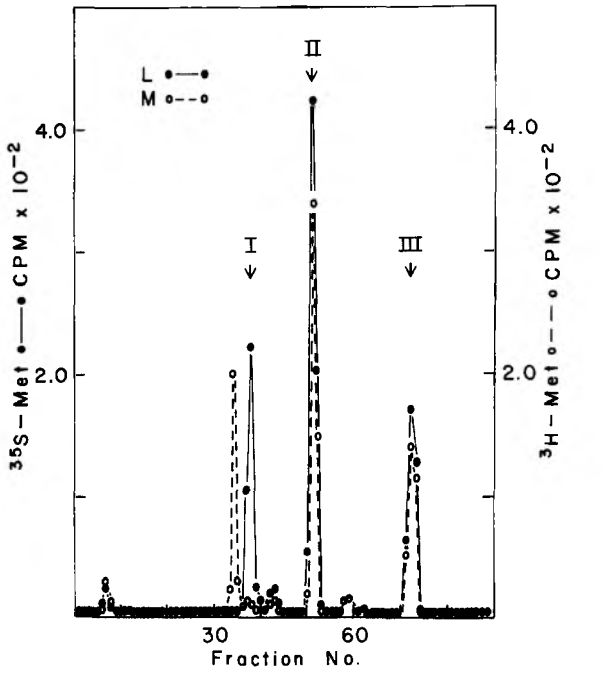

Figure 3. Analysis of the Methionine-Containing Tryptic Peptides of HGPRT Isolated From L$^+$ and Mutant (M) Cell Extracts

L$^+$ and mutant cells were labeled with ^{35}S- and ^{3}H-methionine, respectively. The wild-type and mutant HGPRT was purified from cell extracts by immune precipitation and SDS-urea polyacrylamide gel electrophoresis. The purified proteins eluted from the gels were mixed, digested with TPCK-treated trypsin and analyzed as described in Figure 2.

Processing of HGPRT?

As previously mentioned, the majority of HGPRT$^-$ CRM$^+$ mutants have subunit molecular weights indistinguishable from wild-type protein. We have isolated mutants with altered subunit molecular weights however, and while some are smaller, others are larger than wild-type HGPRT. Examples of such mutants are illustrated in Figure 8. The CRMs shown migrate respectively faster than, the same as and slower than wild-type HGPRT on calibrated SDS-urea polyacrylamide gels. The altered migration of the mutants could reflect altered levels of post-synthetic chemical modification of HGPRT (carbohydrate addition, phosphorylation, adenylation and so on). To date, however, we have failed to generate any data which would support the idea that HGPRT is post-synthetically chemically modified. We therefore believe that the altered mobilities of the CRMs in SDS-urea polyacrylamide gels reflect altered subunit molecular weights of the mutant proteins. We believe that most of these mutants are not nonsense mutants for a number of reasons. First, some are clearly larger than wild-type HGPRT. Second, some of the revertants of

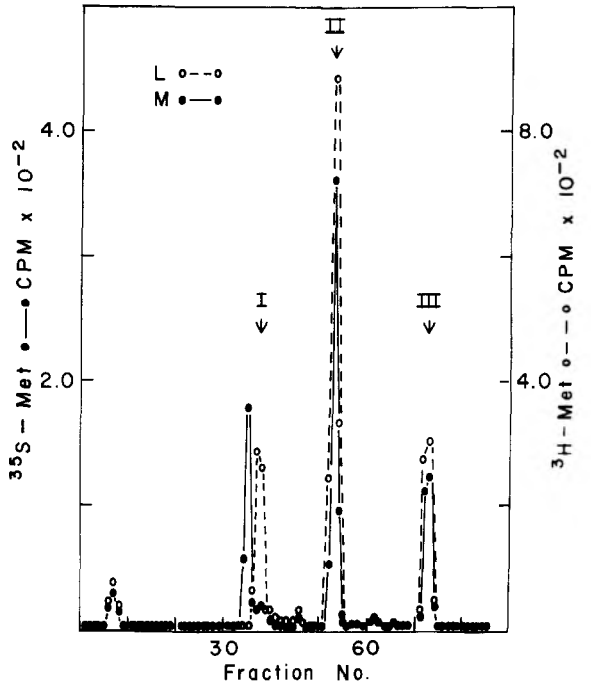

Figure 4. Further Analysis of the Methionine-Containing Tryptic Peptides of HGPRT Isolated From L$^+$ and Mutant (M) Cell Extracts

The experiment shown in this figure was as described in Figure 5, except that the radioactive labels were reversed.

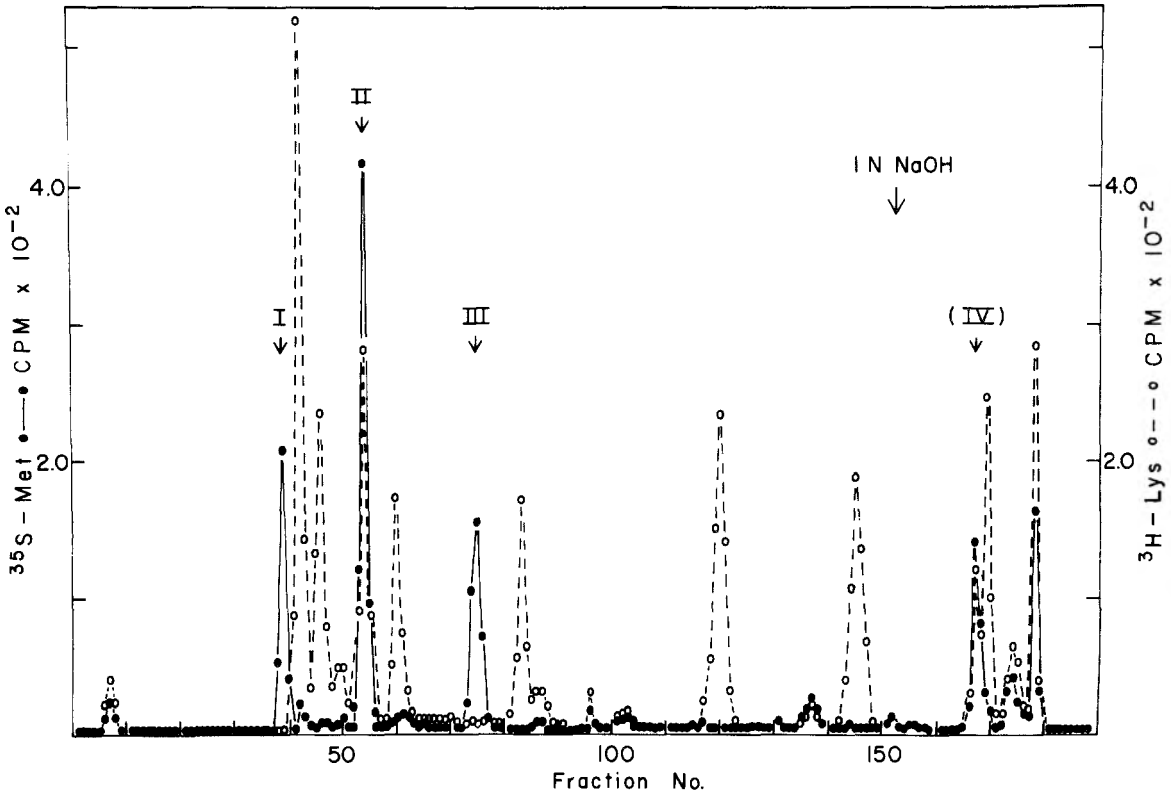

Figure 5. Analysis of the Lysine- and Methionine-Containing Tryptic Peptides of HGPRT

Mouse L^+ cells were separately labeled with ^{35}S-methionine and ^{3}H-lysine. The labeled HGPRT was purified and trypsinized, and the peptides were analyzed as described in Figure 2. The methionine-containing peptides II and IV also contain lysine. A nonmethionine-containing lysyl peptide elutes just after peptide IV. The reasons for believing that peptide IV is a legitimate tryptic peptide of HGPRT are that it is always found to be present in a ratio of one to one relative to peptides I and III, and that the ratio of methionine to lysine in peptide IV is 1 and not <1. The radioactive material beyond peptide IV, which requires 1 N NaOH for elution, may represent more complex tryptic products (that is, unresolved peptides or polypeptides which have not been completely hydrolyzed by trypsin).

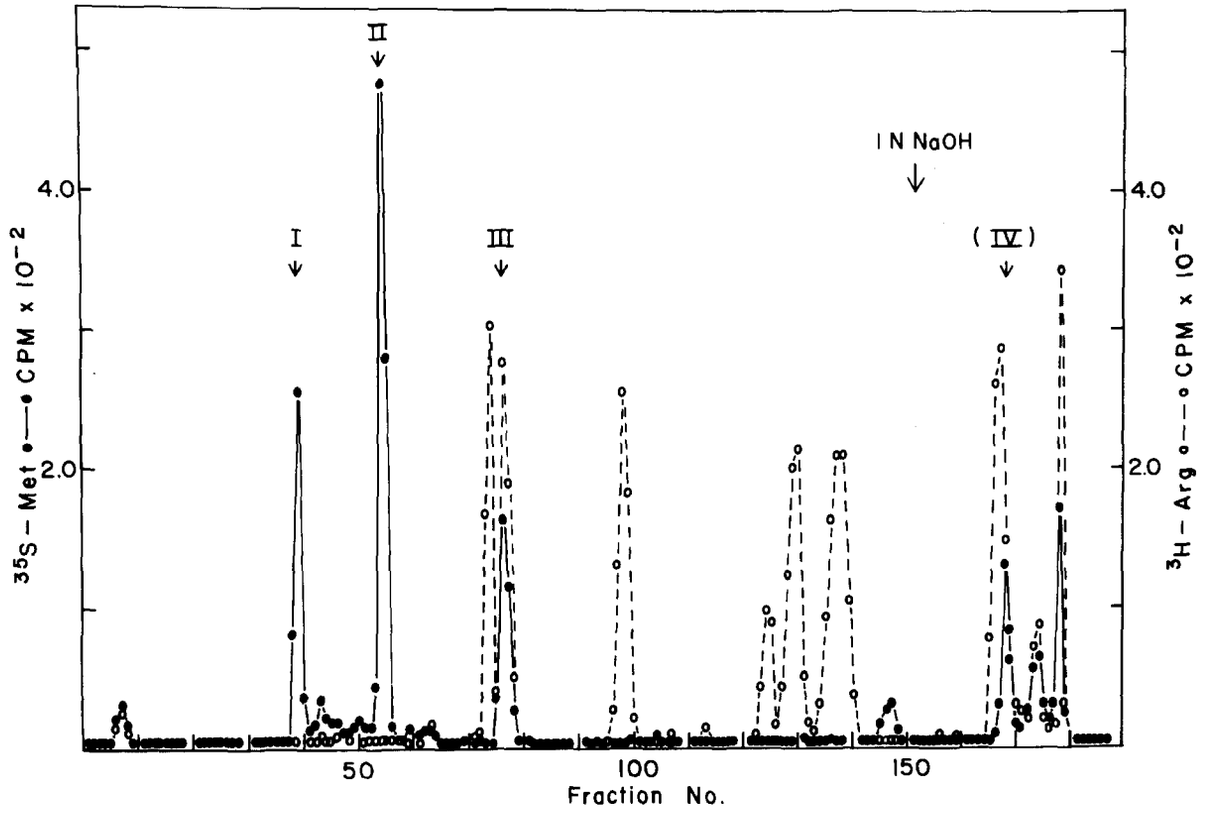

Figure 6. Analysis of the Arginine- and Methionine-Containing Tryptic Peptides of HGPRT

The analysis is as described in Figure 5, except that mouse L^+ cells were labeled with ^3H-arginine instead of ^3H-lysine. A nonmethionine-containing arginyl tryptic peptide elutes just ahead of peptide IV. Peptide III is observed to contain arginine.

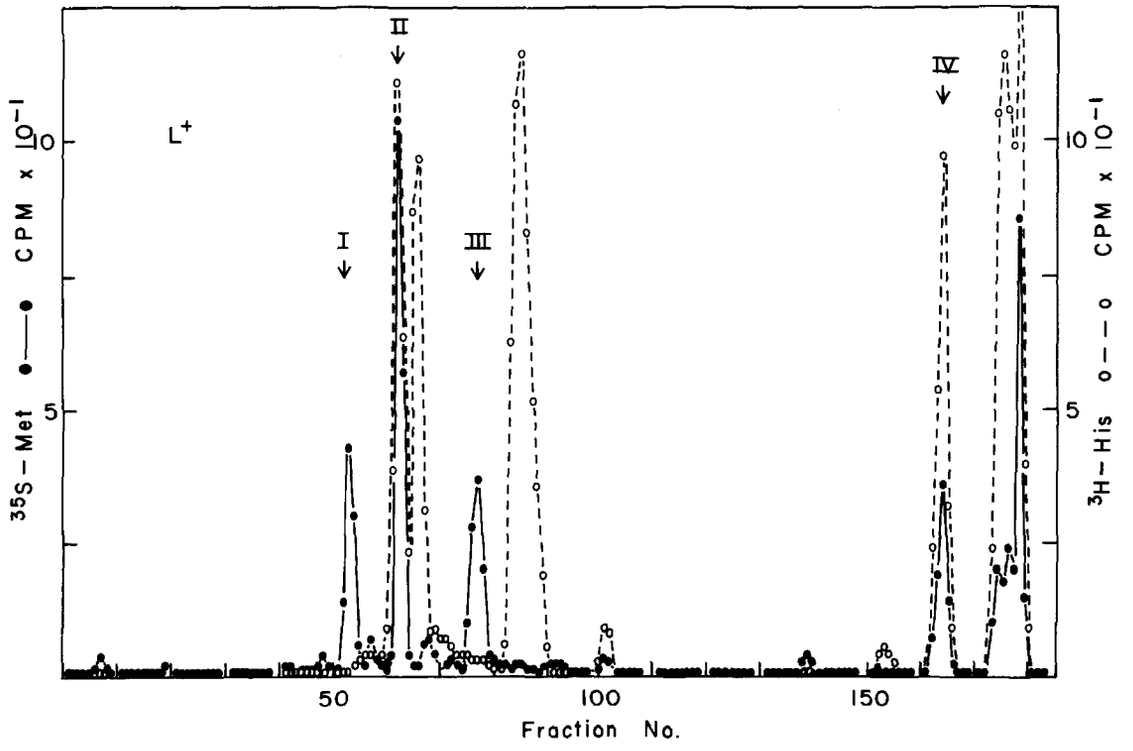

Figure 7. Analysis of the Histidine- and Methionine-Containing Peptides of HGPRT

The analysis is as described in Figure 5, except that mouse L^+ cells were labeled with ^3H-histidine instead of ^3H-lysine. It is noted that peptide I does *not* contain histidine.

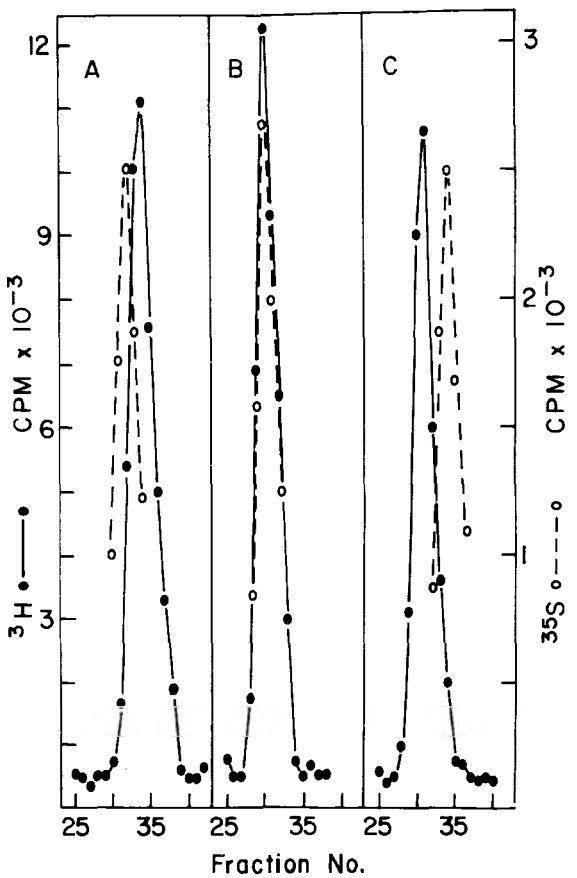

Figure 8. Analysis of the Electrophoretic Mobility of Three HGPRT⁻, CRM⁺ Mutants in SDS-Urea Polyacrylamide Gels

Mouse L⁺ cells were labeled with ^{3}H-lysine and HGPRT⁻ CRM⁻ mutant cells with ^{35}S-methionine. The wild-type and mutant HGPRT molecules were purified and subjected to electrophoresis in SDS-urea polyacrylamide gels as described in Figure 1.

such mutants retain their altered subunit molecular weight. Third, they are not sensitive to phenotypic suppression. Rather, we believe that many of these mutants with altered subunit size represent structural gene mutants, which as a result of the mutation have acquired alternate processing sites. Proof of this hypothesis will require primary structural analysis of the affected proteins.

One of the reasons for introducing the topic of processing of HGPRT is to point out the dangers of relying solely on altered subunit molecular weight as a criterion for a nonsense mutation and to emphasize the need to develop additional criteria. The approach which we have used to provide a second criterion is to introduce, by microinjection, suppressor tRNA isolated from bacteria and yeast into the mutant cell line and to look for restoration of HGPRT activity (that is, a type of phenotypic suppression).

Microinjection Experiments

Schlegel and Rechsteiner (1976), as well as Loyter, Zabai and Kulka (1975) have developed a method for microinjection of macromolecules into mammalian cells in culture. The method involves fusing red blood cells preloaded with macromolecules to the mammalian cells using ultraviolet-inactivated Sendai virus. The loading of the red blood cells with macromolecules is accomplished by hypotonic hemolysis of the red blood cells in the presence of the desired macromolecules. The details of loading and fusing of the red blood cells differ as described by the two groups. We have more closely followed the procedures of Schlegel and Rechsteiner (1975).

To be certain that microinjection was achieved under our experimental conditions, we injected HGPRT molecules into the mutant cell line. The macromolecules within the red blood cells were removed by a prelysis step; the cells were then resealed, loaded with HGPRT and fused to the HGPRT⁻ cell line (Figure 9B). For comparison, red blood cells not loaded with HGPRT were also fused with the same cell line (Figure 9A). HGPRT activity present in the two populations of mouse L cells after fusion was determined by incubating the cells with ^{3}H-hypoxanthine, fixing the cells, washing the fixed cells with 10% TCA to remove the unincorporated hypoxanthine and analyzing the level of incorporation by autoradiography. The cells which were fused with HGPRT-loaded red blood cells have approximately 10 fold greater density of exposed silver grains overlying them. The grain density over the control cells (Figure 9A) is typical for this cell line under comparable incubation and exposure conditions whether or not the cells have been fused with unloaded red blood cells.

Assuming a Poisson distribution, an analysis of the number of null cells (those with low grain density, suggesting that they had not successfully fused with any HGPRT-loaded red blood cells) indicated that on the average, we were fusing one loaded red blood cell per fibroblast cell.

Table 1 gives a quantitative analysis of the HGPRT microinjection experiment. The HGPRT activity loaded into the red blood cells is given in units of concentration (activity per unit vol) relative to the activity in wild-type mouse L cells. The amount of HGPRT activity transferred to the mutant cell line was proportional to the amount of HGPRT loaded into the red blood cells. The volume of a red blood cell was approximately 1% of the volume of a mouse fibroblast cell. Thus the amount of HGPRT activity transferred was consistent with the fusion, on the average, of one red blood cell per fibroblast cell.

When we were confident that we could microinject HGPRT into the mutant cell line, we tested the effect of microinjecting suppressor tRNAs into our mutant cell line. Figure 10 gives the results of microinjecting partially purified tRNA from three iso-

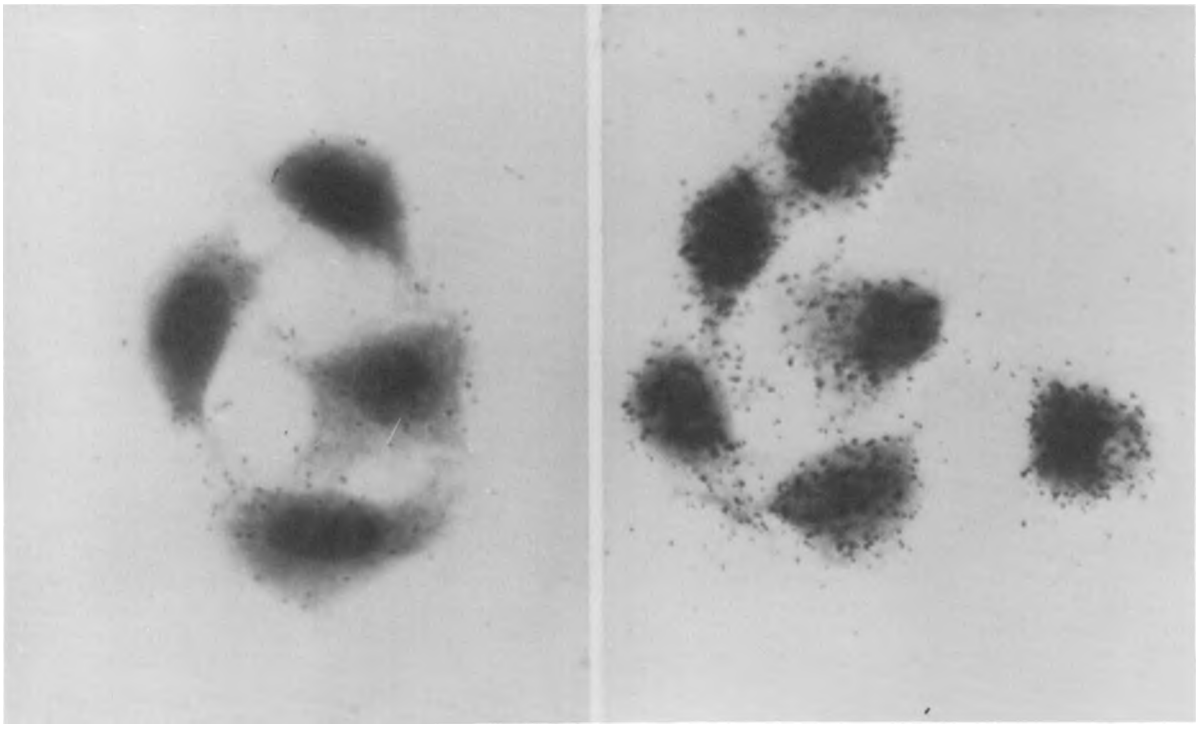

Figure 9. Autoradiographic Analysis of the HGPRT Activity Present in HGPRT$^-$ Mouse L Cells After Fusion with Red Blood Cells Containing (B) and Not Containing (A) HGPRT Molecules

Human red blood cells were hypotonically lysed in the presence of a 20 fold excess of buffer to remove internal macromolecules, resealed and loaded with purified HGPRT (B) and bovine serum albumin (A). The loaded red blood cells were fused to the HGPRT$^-$ cell line using ultraviolet-inactivated Sendai virus. After fusion, the cells were plated onto glass coverslips and incubated at 37°C for 36 hr. HGPRT activity was then measured by incubating the cells with ^{3}H-hypoxanthine, fixing the cells with methanol, washing the cells with 10% TCA and overlaying the cells with autoradiographic emulsion. The autoradiograms were exposed in the dark for 72 hr and developed.

Table 1. Microinjection of HGPRT into the HGPRT⁻ Mouse L Cell Line

HGPRT Loaded into Red Blood Cells	^{3}H-Hx Incorporated by HGPRT⁻ Fibroblast After Fusion (CPM)	% L Cell Activity
0	844	0.08
1X	9,940	1.0
2X	20,720	2.0

Human red blood cells were hypotonically lysed in the presence of a large excess of buffer to remove the internal macromolecules and resealed. The red blood cells were then loaded with three concentrations of purified HGPRT (0, 1X and 2X). The units of HGPRT are given as the concentration present in L⁺ cells. The HGPRT activity transferred to the mutant L cell line was determined by measuring in culture the incorporation of ^{3}H-hypoxanthine into TCA-precipitable product. The ratio of endogeneous human HGPRT activity to the loaded mouse HGPRT in the 1X and 2X red blood cells was 1/14 and 1/28, respectively.

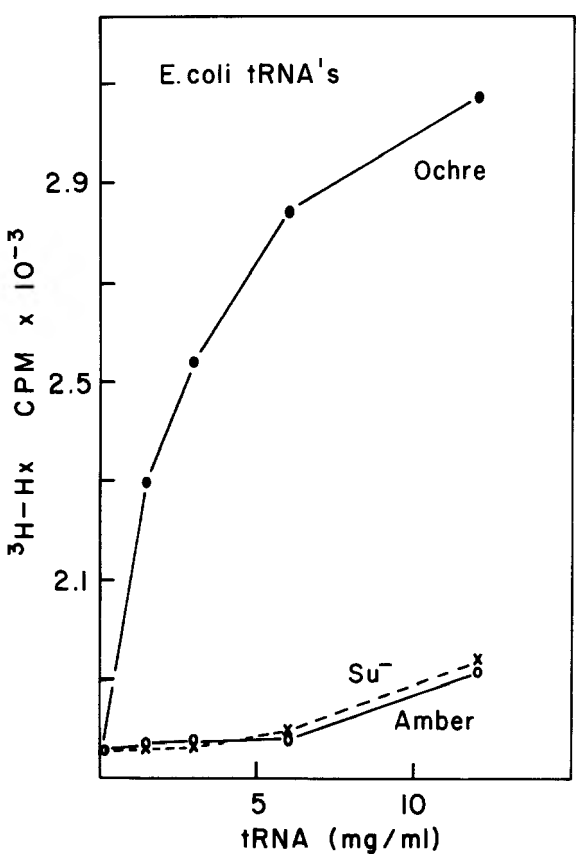

Figure 10. Restoration of HGPRT Activity to the HGPRT⁻ Cell Line by Microinjection of E. coli Ochre-Suppressor tRNA

Human red blood cells, loaded with increasing concentrations of tRNA, were fused to the mutant L cell line using ultraviolet-inactivated Sendai virus. The tRNA was isolated from three strains of E. coli: S26, S26R1E and PS2 which contain no suppressor, an amber-suppressor and an ochre-suppressor, respectively. The units of tRNA along the abscissa are given as the concentration of tRNA loaded into the red blood cells. HGPRT activity after fusion was measured in culture by determining the level of ^{3}H-hypoxanthine incorporation into a TCA-precipitable product.

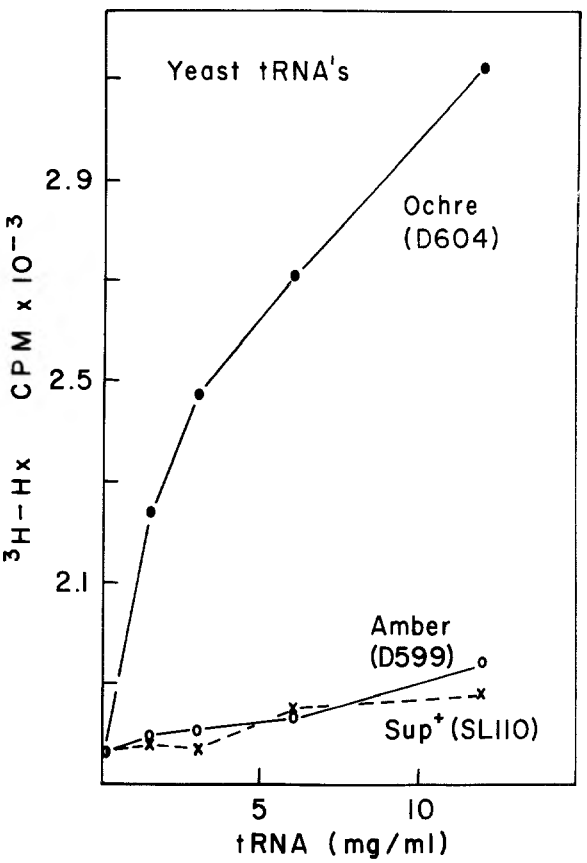

Figure 11. Restoration of HGPRT Activity to the HGPRT⁻ Cell Line by Microinjection of Yeast Ochre-Suppressor tRNA

The experimental details were as described in Figure 10, except that the sources of tRNA were three related strains of yeast: SL110-4D, D599-4B and D604-7D containing no nonsense suppressor, an amber-suppressor and an ochre-suppressor, respectively.

genic strains of E. coli into the mutant cell line. The strains were S26 (the suppressor-minus parental strain), S26RIE (which contains an Sul amber suppressor) and PS2 (which contains the corresponding ochre-suppressor). The tRNAs were purified using benzoylated-DEAE-cellulose chromatography as previously described (Capecchi et al., 1975). HGPRT activity was measured 36 hr after fusion with the loaded red blood cells. Microinjection of ochre-suppressor tRNA into the HGPRT⁻ cell line specifically restored HGPRT activity.

Figure 11 shows a similar experiment, except that the sources of tRNAs were three closely related strains of yeast, S. cerevisiae. We observed again that only the yeast ochre-suppressor tRNA restores HGPRT activity to the mutant cell line.

Discussion

We have described an HGPRT⁻ mutant cell line derived from mouse L cells with the following properties: first, it contains approximately 0.08% of the

HGPRT activity present in the parental cell line; second, it is CRM^+; third, it has an altered carboxy-terminal tryptic peptide whose chromatographic behavior is consistent with this peptide being shorter than the corresponding wild-type peptide; fourth, the mutant can be phenotypically corrected by microinjecting into the cells ochre-suppressor tRNA from either bacteria or yeast, but not by injecting amber-suppressor or wild-type tRNA from the same sources. From these properties, we conclude that this mutant contains an ochre nonsense mutation in the HGPRT structural gene.

The obvious next step is to analyze a series of revertants of this mutant for suppressor tRNA activity. For this purpose we have developed in vitro suppressor assays using mammalian protein-synthesizing components (Capecchi et al., 1975; unpublished results). The mutant does revert to $HGPRT^+$ at a frequency of 10^{-7} after mutagenesis.

A number of investigators have reported shortened mutant gene products in higher eucaryotes and their viruses. Epstein, Waterson and Brenner (1974) have reported a mutant nematode which contains a shortened myosin peptide. Adetugbo, Milstein and Secher (1977) have described a mutant myeloma cell line which synthesizes a shortened immunoglobulin H chain, and Summers, Wagner and Summers (1975) have reported Herpes simplex viral mutants containing shortened thymidine kinase peptides. Some of these mutants have subsequently been shown to have been generated by internal deletions or frameshift mutations. Most recently, R. F. Gesteland and his co-workers have isolated nonsense mutants in an SV40-adeno virus hybrid (personal communication). These investigators can identify the cell-free translation product of the affected gene and suppress it in vitro by the addition of the appropriate suppressor tRNA.

Experimental Procedures

The methods used for culturing the cells, labeling the cells with radioactive precursors, preparing the cell extracts, immunoprecipitating HGPRT, processing the immunoprecipitates and electrophoresing the immunoprecipitates on SDS-urea polyacrylamide gels have been described in detail elsewhere (Sharp et al., 1973; Wahl et al., 1975; Capecchi et al., 1975).

Trypsin-Digestion of HGPRT

Wild-type L^+ and mutant cells were grown to 75% confluency on 100 mm plates containing MEM plus 10% fetal calf serum. The medium was removed and replaced with 3.0 ml of MEM containing 100 μCi/ml of the desired radioactively labeled amino acid (spec. act. approximately 20 mCi/μmole). The cells were then incubated with gentle shaking for 24 hr at 30°C. The isotopically labeled HGPRT molecules were purified from cell extracts by immunoprecipitation and SDS-urea polyacrylamide gel electrophoresis. The gels were cut into 1 mm slices, and the HGPRT was eluted from the gel slices by gently shaking them in 0.6 ml of 0.01% SDS for 36 hr at 30°C. The fractions containing the labeled HGPRT were determined by counting a 20 μl aliquot of each supernatant in a Packard Tri-Carb scintillation counter. The appropriate fractions (1–2 per gel) were pooled and lyophilized. SDS was removed by resuspending the samples in 150 μl of water, precipitating the protein with 25% TCA and washing the precipitates twice with 1 N HCl. The samples were then resuspended in 100 μl of a 1% ammonium bicarbonate solution (pH 8.1), and 3 μg of trypsin (Worthington-193 μ/mg) were added. After the samples had incubated for 2 hr at 30°C, 50 μl of the bicarbonate solution and 2 μg more trypsin were added. Trypsin digestion was continued for an additional 12 hr at 30°C. The ammonium bicarbonate was removed from the samples by lyophilization.

Analysis of the HGPRT Tryptic Peptides

The tryptic peptides of HGPRT were fractionated by cation-exchange chromatography at 50°C on an Aminex A-5 (Bio Rad) column. The lyophilized tryptic digests were resuspended in 100 μl of H_2O, acidified by adding 5 μl of formic acid and loaded onto a 2 mm × 150 mm column. The samples contained from 2000–10,000 cpm. The flow rate was maintained at approximately 4 ml/hr using a Milton Roy minipump at a pressure of approximately 400 psi. After the sample was loaded, 2.0 ml of 0.1 M pyridine acetate (pH 3.5) were passed through the column. The peptides were eluted using a 28 ml linear pyridine acetate gradient [from 0.1 M (pH 3.5) to 1.1 M (pH 5.2)]. At the end of the gradient, the column was washed with 2.0 ml of 2 M Tris (pH 8.0) and 8.0 ml of 1 N NaOH. 0.2 ml fractions were collected. The radioactivity in each fraction was determined by adding 2.5 ml of scintillation fluid and counting the solution in a Packard Tri-Carb counter.

Microinjection of HGPRT into the Mutant Cell Line

10 ml of human blood were collected into a heparin-treated vacutainer to prevent coagulation. The serum and white blood cells were carefully removed during four washes with Hank's solution. The red blood cells were preswollen by resuspending 0.5 ml of packed cells in 5.0 ml of 60% Hank's. The swollen red blood cells were centrifuged at 650 × g for 12 min, and the supernatant was carefully removed. The endogenous level of HGPRT in the red blood cells was reduced by the following procedure: 0.5 ml of loading buffer [0.01 M Tris (pH 7.4), 0.005 M DTT] and 2.5 ml of 25% Hank's were added to 0.5 ml of preswollen red blood cells; the mixture was vortexed and incubated at 0°C for 2.5 min. After incubation, hemolysis was stopped by adding 400 μl of 10× Hank's solution. The cells were then incubated for 1 hr at 37°C and washed 3 times with Hank's. Analysis of the HGPRT content within the red blood cells before and after the hemolysis procedure indicated that the enzymatic activity per cell was reduced 7 fold.

Purified mouse HGPRT was then loaded into these red blood cells by mixing 0.5 ml of packed red blood cells with 0.5 ml of loading buffer containing the desired amount of HGPRT activity. The cells were again incubated for 2.5 min at 0°C, and hemolysis was stopped by adding 125 μl of 10× Hank's. The loaded red blood cells were then incubated for 1 hr at 37°C. After incubation, the cells were washed twice with Hank's solution and once with a buffer of 0.02 M Tris (pH 7.4) and 0.150 M NaCl. Final resuspension of the HGPRT-loaded red blood cells was in 0.02 M Tris (pH 7.4), 0.150 M NaCl and 0.002 M $MnCl_2$. An aliquot of the loaded red blood cells was analyzed for HGPRT content. The amount of HGPRT loaded into the red blood cells was proportional to the amount of HGPRT present in the loading buffer during the hemolysis step.

Fusion of the loaded red blood cells with the $HGPRT^-$ mouse fibroblast cells was accomplished by mixing 2×10^5 mouse cells (in 0.2 ml of 0.02 M Tris, 0.150 M NaCl and 0.002 M $MnCl_2$) with 2×10^7 loaded red blood cells (also in 0.2 ml) and 500 HA units of ultraviolet-inactivated Sendai virus (also in 0.2 ml). The mixture was incubated for 15 min at 0°C followed by a second incubation for 30 min at 37°C.

After fusion, the cells were resuspended in 5 ml of MEM plus 10% FCS and plated onto 18 mm coverslips in a 60 mm petri dish. The cells were then incubated for 36 hr at 37°C in a 5% CO_2 incubator and washed 3 times with warm MEM. The amount of HGPRT activity which was transferred during fusion to the

HGPRT$^-$ mouse cells was determined by measuring the capacity of the cells to incorporate ^{3}H-hypoxanthine into macromolecules. The cells were incubated for 6 hr at 37°C in MEM plus 10% FCS containing 15 μCi/ml of ^{3}H-Hx (spec. act. 10 Ci/mmole). The cells were then washed 4 times with PBS, twice with methanol, once with 10% TCA and twice more with methanol. Autoradiography and direct scintillation counting were used to measure the level of ^{3}H-Hx incorporation. For direct scintillation counting, the coverslips containing the fixed, washed cells were placed into scintillation vials, and 0.5 ml of 0.1% SDS solution were added. After 12 hr at room temperature, 10 ml of scintillation fluid were added, and the samples were counted in a Packard Tri-Carb counter. For autoradiographic analysis, the coverslips containing the fixed, washed cells were dipped in TNB-2 emulsion (Eastman-Kodak) diluted 1:1 with water. Following exposure for 72 hr, the slides were developed for 3 min with Kodak Dectol at 16°C and fixed for 5 min with Kodak Fixer.

As an internal measure of the number of mouse fibroblasts in each experiment, the fibroblasts were prelabeled with a low level of $^{32}PO_4$ (2.5×10^4 cpm/10^6 cells). The cells which were analyzed by autoradiography were not prelabeled with $^{32}PO_4$.

Microinjection of tRNA

The procedure for microinjecting tRNA into the mutant L cells was identical to the methods just described, except that tRNA was substituted in the loading buffer in place of HGPRT. The desired tRNAs were purified approximately 8 fold by chromatography on a benzoylated-DEAE column as previously described (Capecchi et al., 1975). After the red blood cells were loaded with amber- or ochre-suppressor tRNA, an aliquot of the cells was extracted with phenol to recover the tRNA, the aqueous phase was precipitated with 2 vol of ethanol and the precipitates were washed twice with 70% ethanol. This tRNA was assayed for suppressor tRNA activity as previously described (Capecchi et al., 1975). Quantitative recovery of the suppressor tRNA activity was obtained, indicating that equilibrium of the tRNA across the red cell membrane was achieved during the loading procedure, and that tRNA activity was not destroyed within the red blood cells.

Wild-type as well as suppressor tRNA was microinjected into L$^+$ cells at the concentrations indicated in Figures 10 and 11. Neither viability nor HGPRT activity was affected.

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